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Fetal death in cows experimentally infected with *Neospora caninum* at 110 days of gestation

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ABSTRACT

Neospora caninum is a major cause of abortion in cattle, but the reasons why some animals abort and not others remain unclear. Most of the *N. caninum* experimental primary infections in cattle late in gestation, after 120 days of pregnancy, result in birth of full-term congenitally infected fetuses. In the present study, the distribution of parasites and pathogenesis of infection in both dams and fetuses after inoculation with 10^7 culture derived tachyzoites of *N. caninum* NC-Illinois cattle strain at 110 days of gestation were analyzed at 3 weeks, 6 weeks and 9 weeks after infection (WAI) in eight Angus heifers. One dam from the group euthanized at 6 WAI had a dead fetus at necropsy. Extensive lesions were observed in the placenta and tachyzoites were detected in both the placenta and the fetus. The fetus was seropositive and had high IFN-γg production in fetal fluids. Another fetus, still alive when euthanized at 3 WAI, had severe lesions and high IFN-γ production and a similar fate could have been expected if the experimental period would have been longer. Lesions in the placenta of the remaining six dams that had live fetuses at necropsy were mild. In those dams, the fetal and maternal placentas had not separated and contained focal areas of placentitis at the materno-fetal junction. Transplacental infection took place on all fetuses based on detection of parasitic DNA in fetal tissues. The present study shows that experimental *N. caninum* infection of naïve dams after 110 days of pregnancy can lead to fetal death. The results suggest that the severity of placental lesions and the strong IFN-γ response in some fetuses, possibly as part of the immune response trying to control the high parasitemia, might, in fact, be the cause of their death.

1. Introduction

Neospora caninum is an obligate intracellular protozoan parasite that can infect domestic and wild canids, ruminants and horses (Dubey, 2003; Dubey et al., 2007). Abortion and stillbirth due to neosporosis, especially in dairy cattle, have been reported worldwide and *N. caninum* is now considered as one of the most important causes of abortion in cattle (reviewed by Dubey et al., 2006).

Infection in cattle may occur through exogenous transplacental transmission by ingestion of oocysts shed by canids (dogs, coyotes and possibly red foxes), known to be definitive hosts (McAllister et al., 1998; Gondim et al., 2004a; Wapenaar et al., 2006). However, the most common mode of infection and maintenance of infection in cattle herds is vertical or endogenous transplacental

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transmission from infected dams to the fetus across the placenta during pregnancy (Björkman et al., 1996; Paré et al., 1996; Anderson et al., 1997; Schares et al., 1998). Either route of transmission may result in abortion since it has been demonstrated that oral inoculation of pregnant cows with oocysts can also produce abortion (Gondim et al., 2004b). Vertical transmission rates are very high and can occur over several generations and in successive pregnancies and neosporosis can cause repeated abortion (Anderson et al., 1995; Pabón et al., 2007).

The pathogenesis of bovine neosporosis is complex and only partially understood, and the reasons why some animals abort and not others remain unclear (Dubey et al., 2006). Several factors have been reported to affect vertical transmission and occurrence of abortion in *N. caninum* infection, such as timing of primary infection, timing of recrudescence in persistently infected hosts, the effect of pregnancy on maternal immunity, host susceptibility, parasite strain diversity and stage of fetal development at which infection is acquired (Conrad et al., 1993; Barr et al., 1994; Williams et al., 2000; Buxton et al., 2002; Innes et al., 2005).

Experimental inoculation of naïve pregnant cattle in the first trimester can cause fetal death (Dubey et al., 1992; Barr et al., 1994; Williams et al., 2000; Macaldowie et al., 2004; Gibney et al., 2008; Rosbottom et al., 2008). Before 100 days of gestation the bovine fetus may be unable to recognize and respond to pathogens (Osburn et al., 1982). On the other hand, experimental infections in naïve cattle performed at later stages, after 120 days of pregnancy, have resulted in birth of full-term congenitally infected fetuses (Andrianarivo et al., 2001; Williams et al., 2000; Innes et al., 2001; Almería et al., 2003; Maley et al., 2003; Gibney et al., 2008; Rosbottom et al., 2008). However, occurrence of abortion in most naturally infected cattle takes place between 4 and 6 months of gestation (Anderson et al., 1995; Thurmond and Hietala, 1997; Moen et al., 1998; Gonzalez et al., 1999). In chronically infected cattle, *N. caninum* infection prior to pregnancy does not appear to affect the early fetal period, but does have a marked abortive effect after 90 days of gestation, with the highest number of abortions occurring in the second trimester (López-Gatius et al., 2004). A transitory immunosuppression in T lymphocytes starting around 18 weeks of gestation has been demonstrated (Innes et al., 2001), and this could be the cause of the major sensitivity of the animals to parasitemia at that time (Khan et al., 1997; Eperon et al., 1999).

It has been suggested that abortion occurs when primary parasite-induced placental damage jeopardizes fetal survival directly or causes release of maternal prostraglandins that in turn cause luteolisis and abortion. Fetal damage may also occur due to primary tissue damage caused by multiplication of *N. caninum* in the fetus or due to insufficient oxygen/nutrition, secondary to placental damage (Dubey et al., 2006). In addition, maternal immune expulsion of the fetus may occur associated with placental inflammation and the release of maternal pro-inflammatory cytokines in the placenta (Dubey et al., 2006). In some cases, fetal death is less a direct result of parasite replication and more due to the maternal immune response triggered by the parasite (Innes et al., 2005). An essential role of Th1 cytokines, such as IFN-γ and IL-12 in protective immunity against *N. caninum* has been indicated in murine models (Khan et al., 1997; Marks et al., 1998; Baszler et al., 1999). However, during pregnancy a considerable level of immunomodulation may exist (Raghupathy, 1997; Chaouat et al., 2002), and responses such as those induced by IFN-γ can be potentially damaging and may cause rejection or abortion of the fetus. During pregnancy, there appears to be a bias towards type 2 cytokines and away from the type 1 cytokines found to be protective in protozoal infections. It is accepted that abortion due to neosporosis can occur, among other causes, by the shift from beneficial Th2-type towards an excessive Th1-type of immunoresponse during the gestation period, the latter more efficient against *N. caninum* (Quinn et al., 2002; Innes et al., 2005). The immunological control of the parasite in the placenta and/or fetus could be the key to determine the mechanism of abortion (Gibney et al., 2008).

The present study shows that experimental infection of naïve dams late in pregnancy, after 110 days of pregnancy, can cause fatal outcome of pregnancy. The distribution of parasites and the pathogenesis of infection in both dams and fetuses after a primary infection of pregnant heifers with *N. caninum* NC-Illinois cattle-derived strain tachyzoites were analyzed at different times after infection to give some light on the factors that precipitate abortion in some dams and permit transmission without disease in others.

2. Materials and methods

2.1. Animals and infection

Nine Angus heifers seronegative for *N. caninum* (Herd check® anti-*Neospora*, IDEXX laboratories, USA), *Toxoplasma gondii* (modified agglutination test, Dubey and Desmonts, 1987), bovine viral diarrhoea virus (BVDV), infectious bovine rhinotracheitis (IBR) virus and *Leptospira* spp. were used. The cattle were synchronized for oestrus by means of two prostraglandin F2 alpha injections (dinoprost tromethamine, Lutalyse, Upjohn, USA) according to the manufacturer's recommendations. After injection, the heifers were mated by natural service by two bulls and mating activity was determined by the use of Heatmount™ detectors (Kamar Inc., Steamboat Springs, CO, USA). Pregnancy was assessed by rectal palpation 50 days after the last mating. One of the heifers was found not to be pregnant at that time. At approximately 110 days of pregnancy, the rest of the heifers were intravenously (IV) inoculated with 10^7 culture derived tachyzoites of the *N. caninum* Illinois cattle strain (Gondim et al., 2002, generously donated by Dr. M. McAllister).

Cattle were observed daily throughout the experimental period. Rectal temperatures and weight were recorded weekly. Animals with a temperature of $>39.5\,^{\circ}C$ were considered febrile. Animals were euthanized by means of an IV barbiturate overdose at 3 weeks after infection (WAI) (animals C-1302 and C-1304), 6 WAI (animals C-1306, C-1307, C-1314) and 9 WAI (animals C-1310, C-1315, C-1330). The animals were immediately necropsied and subjected to

a full post-mortem examination and tissues removed aseptically. Fetuses were separated from the placenta immediately after euthanization of the heifers.

2.2. Histopathology and immunohistochemistry analysis (IHC)

Paraffin-embedded 5 μm tissue sections were prepared and stained with hematoxilin–eosin (H–E) for histopathological examination or were used for IHC according to Lindsay and Dubey (1989).

Tissues sections from the dams included brain, spinal cord, heart, lung, liver, spleen, skeletal muscle, ovaries, kidney, mesenteric lymph nodes (LN), bronchial LN and uterine LN and nine randomly selected placentomes (three cranial, three medial and three caudal). Fetal tissue sections included brain, spinal cord, heart, lung, liver, spleen, skeletal muscle and inguinal LN.

2.3. Parasite detection by N. caninum-specific PCR (Nc5)

Portions of brain, spinal cord and nine randomly selected placentomes from the dams, as well as brain, spinal cord, lung and liver from the fetuses, were aseptically collected and stored in liquid nitrogen and then at −80 °C until DNA was extracted.

Samples from each tissue were homogenized with a pestle and mortar in liquid nitrogen. DNA was obtained from at least 0.5–1 g of tissue. DNA was extracted as described by Almería et al. (2002). Briefly, after lysis of red blood cells, samples were incubated in proteinase K buffer (200 μg of proteinase K/ml) at 37 °C overnight, phenol extracted and precipitated. For PCR-based diagnosis of *N. caninum* the specific genomic Nc5 region (Kaufmann et al., 1996; Yamage et al., 1996) was selected as the target sequence for DNA amplification. The primers used were Np21-plus and Np6-plus (Liddell et al., 1999). Two sets of samples were extracted for each individual animal tissue and a minimum of two PCR reactions for each set of extractions were performed on separate occasions. The PCR reaction was performed as described by De Marez et al. (1999) using 40 amplification cycles. Amplification products were analyzed by electrophoresis through a 2% agarose gel. DNA extracted from Nc-1 tachyzoites using the High Pure PCR Template Preparation kit (Roche Diagnostic, GmbH, Mannheim, Germany) according to the manufacturer recommendations was used as positive control samples to *N. caninum*. Negative control samples for PCR contaminations were obtained by performing PCR reactions without template DNA. The sensitivity of the reaction has been previously assessed and found able to detect 1 tachyzoite DNA in the background of host DNA (Almería et al., 2002).

2.4. Anti-N. caninum-specific antibodies in infected animals and fetuses

Blood samples were collected weekly from a jugular vein puncture from the infected dams and from cardiac puncture or peritoneal fluids from fetuses at time of necropsy. The serum was separated by centrifugation and 1 ml aliquots of serum were stored at −20 °C until the time of analysis. The sera and peritoneal fluids were tested for *N. caninum*-specific antibodies using a commercial ELISA kits based on the whole tachyzoite lysate of *Neospora* NC-1, according to the manufacturers' instructions (Herd check® anti-*Neospora*, IDEXX Laboratories, USA) with a cut-of value of ≥0.50 as a positive test result.

2.5. Anti-T. gondii specific antibodies

The presence of antibodies to *T. gondii* was tested by the modified agglutination test (MAT) as described previously (Dubey and Desmonts, 1987). Each serum was initially tested at a dilution 1:25, and positive or doubtful sera samples were re-tested at dilutions from 1:25 to 1:1600. A positive control serum diluted from 1:25 to 1:3200 (with a minimum titer of 1:200 in each test) and serum dilution buffer without serum as negative control were included in each test. Titers of ≥1:25 were considered positive.

2.6. Interferon-γ production in fetal fluids

Interferon-γ production levels were measured in serum or peritoneal fluids in fetuses using the Bovigam IFN-γ kit (CSL Veterinary, Victoria, Australia). A standard curve, derived from a series of dilutions of a recombinant bovine IFN-γ standard (rboIFN-γ) was used to quantify levels in the test samples. The mean optical density (OD) values were plotted against the units/ml of rboIFN-γ. A regression line was calculated and the quantity of IFN-γ present in each of the test samples was determined from the standard curve.

3. Results

3.1. Clinical observations

Three of the eight animals inoculated with *N. caninum* were febrile (>39.5 °C) at 2–3 WAI. One of the febrile animals was dam C-1306 that had a dead fetus at necropsy. A slight decrease in weight was observed in all the experimentally infected animals from 1 WAI to 2 WAI but weight gain increased later throughout the experimental period (data not shown). Fetuses from the other infected dams at 3 WAI, 6 WAI or 9 WAI were alive when euthanized.

3.2. Distribution of parasites and lesions in dams and fetuses

N. caninum DNA was detected in the brain in 5 of 8 dams (1 at 3 WAI, 3 at 6 WAI and 1 at 9 WAI), in the spinal cord in 3 of 8 dams (1 at 3 WAI, 2 at 6 WAI and 0 at 9 WAI) and in the placenta of all infected dams (Table 1). In the dam with a dead fetus, *N. caninum* DNA was detected in brain and placental tissues.

N. caninum DNA was observed in all fetuses in at least one of the analyzed tissues. The fetal spinal cord (6 of 8), lung (6 of 6) and brain (5 of 6) were the tissues where the parasite DNA was more frequently found (Table 1). The presence of parasite DNA in the liver was only observed in the fetuses from 3 WAI group, while the presence of DNA in the brain and spinal cord was detected in the 3 fetuses from 9 WAI group. In the dead fetus, DNA was found in the brain and spinal cord (Table 1).

Table 1
Neosporosis in cows inoculated with 10^7 *N. caninum* tachyzoites at 110 days of gestation.

Cow number	WAI	ELISA (cut-off 0.5)		Histopathology cow (placenta)	DNA detection (positive organs)	Fetus ELISA (cut-off 0.5)	Fetus IFN-γ (ng/ml)	Histopathology fetus	DNA detection (organs)
		Day 0	At killing						
C1289[a]	3	ND	ND	ND	ND	ND	ND	ND	ND
C1302	3	0	0.46	++	B, Sc, Pl	0.06	1032	B, H, Li, Lu, Sc, Sk, Sp, T	B, Li, Lu, Sc
C1304	3	0	0.46	None	Pl	0.07	382	No lesion	Li, Lu
C1306[b]	6	0	1.77	+++	B, Pl	0.63[c]	1072	B	B, Sc
C1307	6	0.2	1.03	+	B, Sc, Pl	0.44	57	No lesion	Lu, Sc
C1314	6	0	1.31	+	B, Sc, Pl	0.01	30	No lesion	Lu
C1310	9	0	0.65	+	B, Pl	0.09	44.5	No lesion	B, Lu, Sc,
C1315	9	0.1	1.35	+	Pl	1.88	34.5	B	B, Sc
C1330	9	0	0.78	++	Pl	1.49	44.5	No lesion	B, Lu, Sc

WAI, weeks after infection; +, arbitrary degree of lesions; B, brain; H, heart; Li, liver; Lu, lung; Pl, placenta; Sc, spinal cord; Sk, skeletal muscle; Sp, spleen; T = tongue.
[a] Not pregnant.
[b] Dam with dead fetus at euthanasia.
[c] In this fetus, antibodies and IFN-g were analyzed in peritoneal fluid instead of serum.

Lesions were observed in the placenta of seven of the eight inoculated dams (Table 1). In cows with live fetuses, the fetal and maternal placentas had not separated and contained focal areas of placentitis at the materno-fetal junction. The placentitis was characterized by focal necrosis and infiltration of neutrophils and mononuclear cells, and serum exudation (Fig. 1A). Tachyzoites were identified in placenta of cow 1302 by IHC staining (Fig. 1B). In the cow with the dead fetus there were large areas of necrosis and placentitis with mineralization. Only a few intact tachyzoites and dispersed particulate antigen were seen by IHQ (Fig. 2).

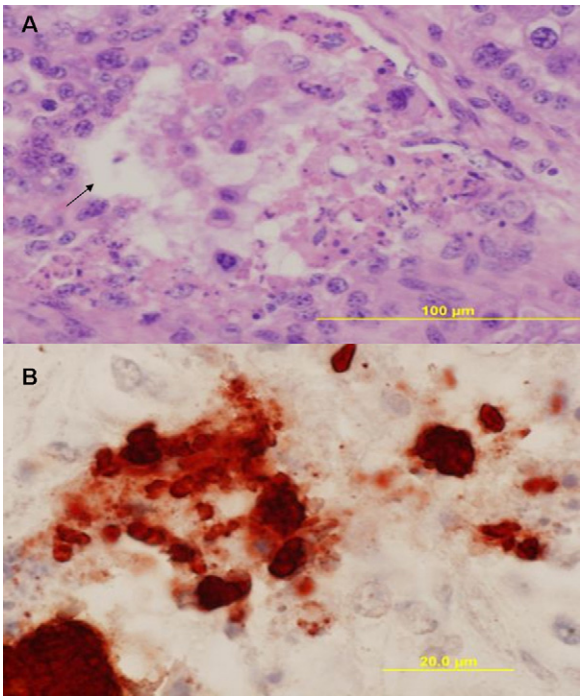

Fig. 1. (A) Focus of necrosis (arrow) and serum exudation at the materno-fetal junction. (B) Materno-fetal placenta with several individual and a group of tachyzoites (all red). Immunohistochemical staining with *N. caninum* polyclonal antibodies.

Lesions were also seen in tissue sections of three fetuses (Table 1). The most extensive lesions were found in the live fetus from the cow C-1302, killed 3 WAI. Inflammatory lesions were identified in several organs but were most severe in the brain, tongue, liver, and the heart (Fig. 3A, B, C and D, respectively). Neural lesions consisted of hemorrhage, mild necrosis, and infiltrations of mononuclear cells. Lesions in other organs were predominantly infiltrations of mononuclear cells. Tachyzoites were identified by IHC only in the brain. Tissues of the dead fetus from cow 1306 had autolyzed but tachyzoites were seen in the brain of the fetus.

3.3. *N. caninum*-specific antibodies in dams and fetus

Detection of *N. caninum*-specific antibodies by ELISA was observed as early as 2 WAI in 2 dams (C-1306, C-1314), one of them the dam with a dead fetus at necropsy. This dam showed the highest levels of antibodies compared to the other dams with antibody levels increasing throughout the experimental period (Fig. 4). All animals were seropositive by 5 WAI.

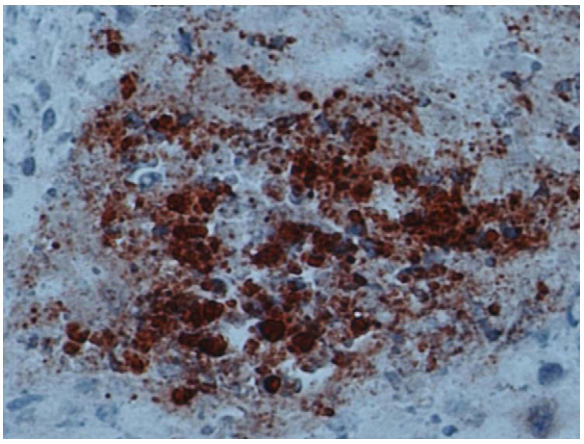

Fig. 2. Immunohistochemical staining of the placenta of the dam that had a dead fetus at necropsy.

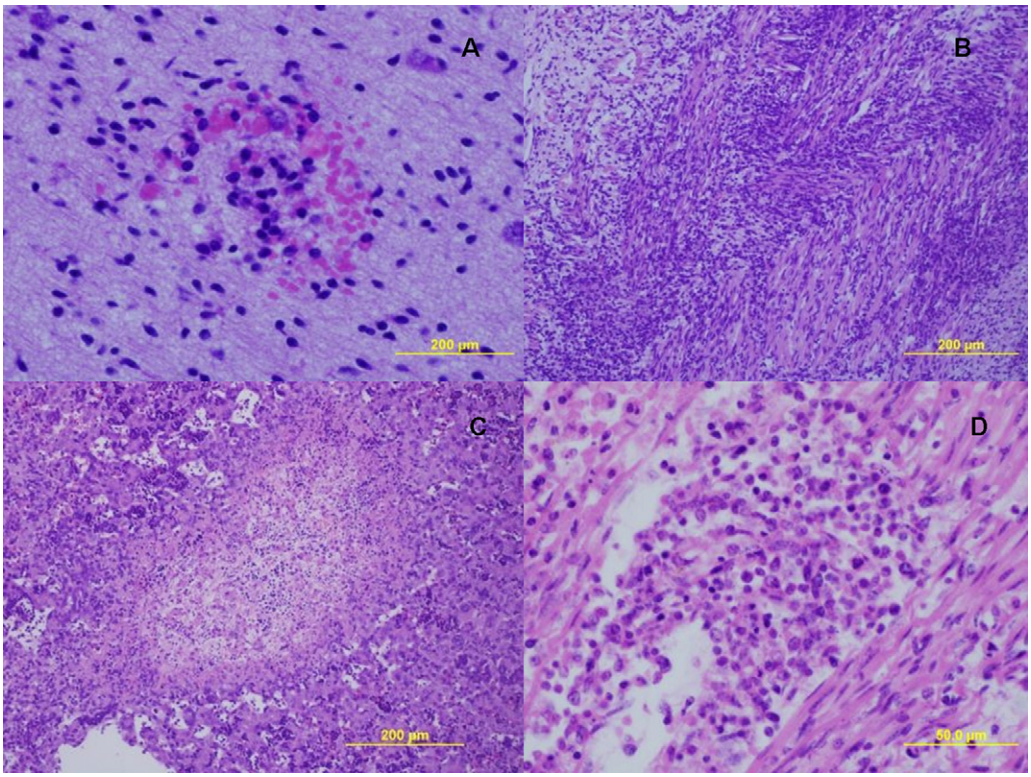

Fig. 3. Lesions in the fetus from cow 1302 euthanized at 3 WAI. (A) Hemorrhage and focal infiltration of mononuclear cells in the brain of fetus from cow 1302. (B) Tongue with severe infiltration of mononuclear cells. (C) Liver with focal necrosis. (D) Heart with an inflammatory focus.

In the fetuses, antibodies were analyzed by ELISA in sera with exception of the dead fetus in which only peritoneal fluid was available. Antibodies did not reach the positive threshold in the 3 WAI group, although levels were close to reach seropositivity. The dead fetus was the only seropositive fetus in the 6 WAI group, while in the group euthanized at 9 WAI, 2 out of 3 fetuses were positive for antibodies against *N. caninum* (Table 1).

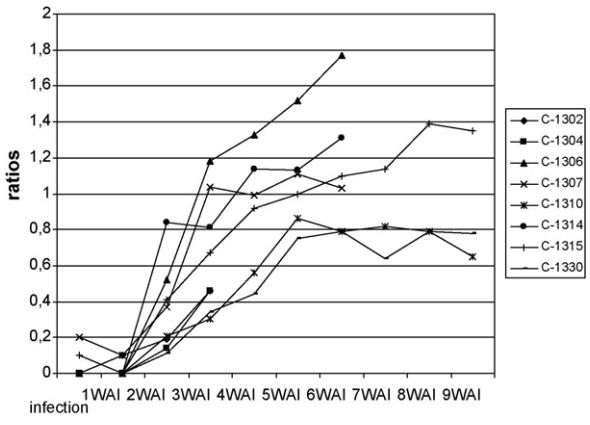

Fig. 4. Dynamics of total IgG antibodies against *N. caninum* in the experimental animals. WAI: weeks after infection. Ratio: optical density (OD) of the sample (S) minus the OD of the negative control (NC), all divided by the OD of the positive control (PC) minus the OD of the negative control ((S − NC)/(PC − NC)).

3.4. IFN-γ production in fetal fluids

IFN-γ production was observed in fetal fluids in both fetuses euthanized at 3 WAI, being especially high in fetus from dam C-1302. In the 6 WAI group, IFN-γ production was only observed, in high levels, in the fluids from the dead fetus and no IFN-γ production was observed in any of the fetuses euthanized at 9 WAI (Table 1).

4. Discussion

The precise causes of *N. caninum* abortion are not known. Some studies have pointed out that the timing of maternal parasitemia, following a primary infection or recrudescence in persistently infected cattle is crucial in determining the fate of the fetus. *N. caninum* infection early in gestation has been related to fetal death (Dubey et al., 1992; Barr et al., 1994; Williams et al., 2000; Guy et al., 2001; Macaldowie et al., 2004; Gibney et al., 2008; Rosbottom et al., 2008) most probably due to lack of fetal immunocompetence. However, the pathogenesis of infection during the second term of gestation, when most abortions occur in naturally infected dams, is less clear. After about 150 days, the fetus is mature enough to recognize and respond to infections (Osburn et al., 1982; Nettleton and Entrican, 1995). In fact, fetuses from experimentally infected cows are able to induce significant cellular immune responses to *N. caninum* infection as early as 4 months of gestational age (Almería et al., 2003).

Similarly, bovine fetal lymphocytes are able to respond to mitogens and alloantigenic stimulation by 120–150 days of gestation (Osburn et al., 1982; Hein et al., 1988; Jensen et al., 1988) and to elicit humoral and cell mediated immune responses when infected with *N. caninum* between 159 and 169 days of gestation (Andrianarivo et al., 2001; Bartley et al., 2004). The present study reports that *N. caninum* experimental infection during the mid-gestation period can also cause abortion.

Fetal death was observed 6 weeks after inoculation of a primary infection with *N. caninum* tachyzoites at 110 days of gestation (152 days of gestation). We also observed severe lesions in a fetus euthanized at 3 WAI and it could be hypothesized that this fetus might have died of disease if the experimental period would have been longer. In a previous study, performed in the same experimental conditions to those in the present study, all fetuses were alive and lesions were scarce when dams were euthanized at 3 WAI (Almería et al., 2003). It was hypothesized then that abortion could have occurred if the experimental period had lasted longer as has been confirmed in the present study. Recently, Gibney et al. (2008) did not observe fetal death in animals infected with *N. caninum* at 210 days of pregnancy, and although the fetuses would likely survive to term, similar to the majority of fetuses in the present study, animals were euthanized at 3 WAI. Our results suggest that longer experimental periods after infection, at least 6 WAI, would be necessary to adequately assess the possibility of occurrence of abortion in experimental infections of pregnant cows with *N. caninum*.

Several factors could be involved in the pathogenesis of fetopathy in the present study. First, the placenta of the dam with the dead fetus was the most extensively damaged and high numbers of tachyzoites were observed in placental tissues. The severity of lesions in the placenta is a crucial factor in the occurrence of *N. caninum*-associated abortion since its ability to sustain the fetus could be impaired (Dubey et al., 2006; López-Gatius et al., 2006; Gibney et al., 2008). Our results highlight the importance of the placenta during infection and its possible role in permitting or limiting spread of infection to the fetus and the occurrence of abortion as indicated by Maley et al. (2003). Dam C1302, whose fetus had severe lesions in multiple organs, also had severe lesions in the placenta, while the rest of dams with live fetuses when euthanized showed less severe lesions even in the presence of parasite DNA, indicating lower parasite damage and/or more controlled immune responses in those animals. In fact, in non-aborting animals the placental lesions, due to crossing by tachyzoites, have been noted to regenerate in experimental infections (Maley et al., 2003).

Although parasitemia was not enough to cause death in the rest of fetuses, vertical transmission took place in all of them as shown by DNA detection, as observed in our earlier study (Almería et al., 2003). Dams experimentally infected 6 weeks prior to mating and challenged at mid-gestation showed protective immunity against vertical transmission (Innes et al., 2001). However, when naïve animals are infected during mid-pregnancy, early in the second trimester, as the present study, or even later, vertical transmission takes place (Williams et al., 2000; Innes et al., 2001; Andrianarivo et al., 2001; Maley et al., 2003; Almería et al., 2003; Gibney et al., 2008). Similarly, in naturally infected animals, immunity acquired in infections prior to pregnancy does not appear sufficient to prevent transplacental transmission to the fetuses. Even when it has been demonstrated that a pre-existing infection can protect against an exogenous challenge, this immunity will not prevent endogenous transplacental transmission (Williams et al., 2003).

It is interesting to note that parasite DNA was detected in the liver of only the fetuses killed early in the infection, at 3 WAI, and there was severe hepatitis in a fetus euthanized at that time. This finding agreed with our previous study (Almería et al., 2003) and from the results of Maley et al. (2003) in experimental infections. In natural infections, hepatic lesions are more prominent and *N. caninum* tachyzoites are higher and more frequent in fetuses from epidemic outbreaks, compared to fetuses from endemic cases (Wouda et al., 1997; Collantes-Fernández et al., 2006). The presence of DNA in the brain and/or spinal cord (CNS) was detected more frequently in the fetuses late after infection (9 WAI) that coincided with previous observations of natural infections in which the parasite persisted in the host, as replicating bradyzoites in tissue cysts, mainly in the central nervous system (Dubey et al., 1998; Collantes-Fernández et al., 2006). The fact that the fetuses at 9 WAI, although vertically infected, were alive indicated that the disease in those fetuses was being controlled and that progression of infection had probably been halted, in agreement with previous studies (Maley et al., 2003, 2006; Bartley et al., 2004).

Some minor clinical signs were observed in the experimental dams. There was a decreased weight gain in the dams between 1 WAI and 2 WAI that could be due to a transitional appetite loss or anorexia after infection, and the inoculation of pregnant cows also caused transient fever in some of the animals. In natural conditions, clinical signs, others than abortion itself, are not generally observed in *N. caninum* infected dams. Even so, experimentally infected cows and newborn calves appear clinically normal with no clinical signs other than fever (Dubey et al., 2006). In the present study, some animals were febrile at some point between 2 WAI and 3 WAI. The increase of temperature took place later and it was more prolonged than that observed in other experimental studies (Maley et al., 2003; Williams et al., 2000).

Although, the presence and replication of the parasite at the materno-fetal interface was a crucial factor in the fetopathy, the immunologically related mechanisms in both dam and dead fetus also showed important differences when compared to the other experimental animals that indicated an important role of such mechanisms in fetal death. On one hand, the dam with a dead fetus showed the highest antibody titers throughout the whole experimental period, with seropositive titers as early as 2 WAI. The higher parasitemia in this dam could have induced such elevated humoral immune response. On the other hand, the dead fetus at 6 WAI was also seropositive and showed IFN-γ production. The immune response in the dead fetus was an indication of an early passage of

tachyzoites after infection. Maley et al. (2003) observed tachyzoites in fetal tissues as early as 14 days post-infection. Similarly, Barr et al. (1994) suggested that tachyzoites cross the placenta and reach the fetus about 10 days after maternal infection. High IFN-γ production, in levels similar to those of the dead fetus, were also observed in the fetus that showed severe lesions in multiple organs, still alive when euthanized at 3 WAI, and this fact could be an indication that this fetus might have died if the experimental period would have lasted longer. Interestingly, neither antibodies nor IFN-γ production were observed in the other fetuses in the 6 WAI group. In addition, 2 of 3 fetuses were seropositive at 9 WAI, but no IFN-γ production was detected at that time. These results suggest that the strong IFN-γ response in the dead fetus, possibly as part of the immune response to the high parasitemia in the fetus may, in fact, have been detrimental for its survival causing sufficient placental and/or fetal pathology to kill the fetus. In experimental *N. caninum* infections in early gestation, that were associated with fetal death, it has been suggested that Th-1 responses in the placenta (resulting in infiltrations of CD4+ T cells, γδ T cells and NK and subsequent production of IFN-γ), are detrimental for the fetus (Maley et al., 2006), and direct damage to the placenta and fetus by multiplying *N. caninum* is likely to have been a contributing factor (Maley et al., 2006). Since other fetuses in the same group did not produce IFN-γ but fetuses at 3 WAI did, the absence of IFN-γ production response in the live fetuses at 6 WAI could indicate that if an earlier IFN-γ production is effective in the control of infection, IFN-γ production is short and the fetus survives, while if IFN-γ production is long lasting or too high, fetal death could occur. It is possible that if lesions in the placenta are not severe, immune responses related to IFN-γ production are sufficient to protect against abortion, and then the placental lesions due to crossing of the placental barrier by tachyzoites could regenerate (Maley et al., 2003).

Recent studies have shown that a Th1 immune response associated to IFN-γ production is linked to protection against *N. caninum* abortion (López-Gatius et al., 2007; Williams et al., 2007). In naturally infected cows, the risk of abortion was 15.6 times higher in seropositive cows not producing plasma IFN-γ than in seronegative animals, whereas neosporosis had no effect in seropositive cows with IFN-γ production (López-Gatius et al., 2007). However, in agreement with the results of the present study, the only dam that had plasma IFN-γ production and aborted in the study of López-Gatius et al. (2007) showed an early and sudden increase (26 times in a 30 days-period) of this cytokine in blood. It has been postulated that an IFN-γ response induced by infection with *N. caninum* during late pregnancy may occur too late to affect and existing, well-establish Th2 response at the maternal–fetal interface (Williams et al., 2000). However, the present study indicated that in experimental infections in the second trimester of gestation if IFN-γ production is too high, abortion can still take place.

Some studies have shown that not only Th1 cytokines (mainly IFN-γ), but also Th2 cytokines are upregulated in the course of *N. caninum* experimental infections in aborted and non-aborted dams (Almería et al., 2003; Rosbottom et al., 2007, 2008). Further analysis of cytokine gene expression (Th1, Th2 and regulatory cytokines) in the *N. caninum* infected dams and fetuses needs to be performed to help elucidate which immune responses are related to abortion and/or to transplacental infection during the course of infection.

In conclusion, the present study shows evidence of fetopathy in an animal experimentally infected after 110 days of gestation. Our results suggest that lesions in the placenta and fetus and the related immunological mechanisms, in particular, the strong IFN-γ response in some fetuses, could be more important that the timing of experimental infection for abortion to take place in *N. caninum* infected animals.

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